BRCA1, BRCA2 MUTATIONS AND THE ASSOCIATION WITH THE CLINICOPATHOLOGICAL CHARACTERISTICS OF WOMEN WITH EARLY-ONSET BREAST CANCER

By:

DR MOHAMAD BAZLI B GHAZALI

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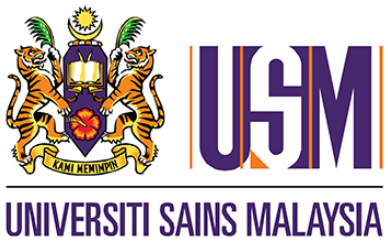

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LIST OF ABBREVIATIONS

AE	elution buffer
AF2	Activation function 2
AL	lysis buffer
ALH	atypical lobular hyperplasia
AMDI	Advance Medical and Dental Institute
ATM	Ataxia telangiectasia mutated
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
bp	Base Pairs
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
BRCT	BRCA1 C Terminus domain
cDNA	Complementary Deoxyribonucleic Acid
CHEK2	checkpoint kinase 2
CI	confidence interval
CIN	chromosomal instability
CRF	clinical report forms
CtIP	CtBP-interacting protein
DCIS	ductal carcinoma in situ
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DSB	double strand break
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ER	estrogen receptor

ER1	estrogen receptor 1
ERBB2	Receptor tyrosine-protein kinase erbB-2
ER-α	estrogen receptor alpha
EtBr	Ethidium Bromide
FA	Fanconi anemia
FGFR2	Fibroblast growth factor receptor 2
FISH	Fluorescence In Situ Hybridization
HBOC	hereditary breast and ovarian cancer
HE-M	heterozygous
HER2	human epidermal growth factor receptor 2
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HO-M	homozygous mutant
HO-W	homozygous wildtype
HPE	histopathological examination
HR	hazard ratio
HRT	Hormone Replacement Therapy
HUSM	Hospital Universiti Sains Malaysia
IDC	intra-ductal carcinoma
ILC	Invasive lobular carcinoma
IQR	Interquartile range
KS	Kolmogorov-Smirnov
LB	lithium borate buffer
LCIS	lobular carcinoma in situ
mRNA	messenger ribonucleic acid
MS	missense
NCBI	National Centre for Biotechnology Information

NCR	National Cancer Registry
NG	base substitution at complete genomic nucleotide position
NM	base substitution at the mRNA level
NOS	not otherwise specified
NST	no special type
OR	odd ratio
PAR	poly-ADP ribose
PCR	Polymerase Chain Reaction
PR	progesterone receptor
PS	Power and Sample Size software
rpm	Rotation Per Minute
RU	Research University grant
RW1	RNeasy Washing Buffer
RW2	RNeasy Washing Buffer
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
ssDNA	single-strand DNAs
SYN	synonymous
TNM	Classification of Malignant Tumours
TP53	Tumour Protein 53
USM HREC	USM Human Research Ethics Committee
UV	ultraviolet light
WHEL	Women's Healthy Eating and Living
WHI	Women's Health Initiative
WINS	Women's Intervention Nutrition Study

LIST OF SYMBOLS

%	percentage
/	or
:	ratio
<	less than
=	equal to
>	more than
®	trademark registered
µl	mikrolitre
µm	mikrometre
µM	micromolar
1U/µl	unit per microlitre
1-β	statistical power
A	adenosine (base)
A_{260}/A_{280}	the optical spectrometer measurement of absorbance
bp	basepair
C	cytocine (base)
F	forward (primer)
G	gauge
G	guanine (base)
g	gram
H_2O	water
m	ratio of control / cases
M_1	initial concentration
M_2	final concentration

min	minute
ml	mililitre
mm	milimetre
mM	milimolar
n	number of subjects
ng/µL	nanogram per microlitre
ºC	degree celcius
p	p value
Pa	probability of exposure in cases
Po	probability of exposure in controls
q	1-prevalence
R	reverse (primer)
sec	second
T	tiamine (base)
Ta	annealing temperature
™	trademark unregistered
V	volt
V_1	volume needed
V_2	volume sample
vs	versus
α	type 1 error
Δ	precision
λ	lambda (wavelength)

MUTASI-MUTASI *BRCA1*, *BRCA2* DAN HUBUNGKAITAN DENGAN CIRI-CIRI KLINIKOPATOLOGIKAL DI KALANGAN WANITA YANG MENGHIDAP BARAH PAYUDARA PADA USIA AWAL.

ABSTRAK

Pengenalan: Mutasi *BRCA1* dan *BRCA2* telah dikenalpasti mempunyai hubung kait dengan kanser payudara pada usia awal dan ciri-ciri klinikal dan patologi yang buruk. Setakat ini, terdapat kekurangan dalam penyelidikan yang mengkaji hubungan antara jenis-jenis mutasi *BRCA1/2* dan ciri-ciri klinikal dan patologi barah payudara di Malaysia. Oleh itu, tujuan kajian ini adalah untuk memastikan sama ada terdapat perbezaan antara jenis-jenis mutasi *BRCA1/2* dari segi ciri-ciri klinikal dan patologi barah payudara di kalangan wanita-wanita yang mengalami penyakit ini pada usia awal.

Metodologi: Tujuh puluh wanita berusia 40 dan ke bawah yang telah didiagnosa dengan barah payudara dan menjalani pemeriksaan susulan di Hospital Seberang Jaya, Pulau Pinang, telah direkrut untuk kajian ini. Ciri-ciri klinikal (umur, etnik, rawatan adjuvan, sejarah keluarga berkenaan kanser payudara dan ovary) dan patologi (reseptor untuk estrogen, progresteron, HER2 dan ketumbuhan yang negative untuk kesemua jenis reseptor, peringkat dan gred ketumbuhan) telah diperolehi secara retrospektif daripada penilitian rekod perubatan. Tiga mililiter darah telah diambil daripada setiap subjek yang kemudiannya digunakan untuk

pengekstrakan DNA. DNA ini kemudian diskrin untuk mutasi titisan germa bagi gen *BRCA1* (exon 11, 13 dan 16) dan *BRCA2* (exon 10 dan 11) dengan menggunakan tindakbalas berantai polymerase spesifik alel.

Keputusan: Kelaziman mutasi *BRCA2* sahaja dan mutasi campuran *BRCA1* dan *BRCA2* adalah 28.6% (95% selang keyakinan: 18.3%, 39.2%) dan 71.4% (95% selang keyakinan: 60.8%, 80.2%). Tiada *BRCA1* atau *BRCA2* gen jenis liar dan mutasi pada *BRCA2* sahaja didapati dikalangan subjek kajian ini. Tiada hubungkait yang ketara antara jenis mutasi *BRCA* (*BRCA2* sahaja dibandingkan dengan kombinasi mutasi *BRCA1* dan *BRCA2*) daripada segi ciri-ciri klinikal dan patologi ketumbuhan payudara. Walaubagaimanapun, tiga mutasi *BRCA1* (3232A>G (rs16941, exon 11), 3667A>G (rs16942, exon 11) dan 4427T>C (rs1060915, exon 13) mempunyai hubungkait ketara dengan kumpulan saiz ketumbuhan peringkat lanjut (nilai p = 0.032, 0.049 dan 0.043). Selain itu, mutasi pada posisi 3232A>G mempunyai hubungkait ketara dengan ketinggian tahap risiko untuk ketumbuhan payudara yang negatif untuk reseptor HER2 (nisbah ganjil: 7.50 (95% selang keyakinan: 1.439, 39.089), nilai p = 0.017) dan yang berstatus negatif untuk kesemua reseptor hormon (nisbah ganjil: 4.375 (95% selang keyakinan: 1.193, 16.038), nilai p =0.042). Tiada hubungkait ketara didapati antara genotip mutasi *BRCA2* dan ciri-ciri klinikal dan patologi ketumbuhan payudara.

Kesimpulan: Kombinasi mutasi *BRCA1* dan *BRCA2* mempunyai kelaziman tertinggi, diikuti mutasi *BRCA2* sahaja. Tiga mutasi titisan germa *BRCA1* mempunyai hubungkait ketara dengan kumpulan saiz ketumbuhan peringkat lanjut manakala hanya satu mutasi *BRCA1* mempunyai hubungkait ketara dengan

ketumbuhan yang negatif untuk reseptor HER2 dan berstatus negatif untuk kesemua reseptor hormon. Walaubagaimanapun, lebih banyak kajian diperlukan untuk menambahbaik kekurangan-kekurangan yang dihadapi dalam kajian ini.

BRCA1, BRCA2 MUTATIONS AND THE ASSOCIATION WITH THE CLINICOPATHOLOGICAL CHARACTERISTICS OF WOMEN WITH EARLY-ONSET BREAST CANCER

ABSTRACT

Introduction: *BRCA1* and *BRCA2* mutations have been associated with early-onset breast cancers and adverse clinico-pathological features. To date, there is paucity of studies in Malaysia investigating the relationship between types of *BRCA1/2* mutations and clinicopathological characteristics of breast cancers. This study therefore aims to ascertain whether there are differences between different types of *BRCA1/2* mutations in terms of clinico-pathological attributes of breast cancers amongst females with early-onset breast cancers in Malaysia.

Methodology: Seventy females aged 40 or less with confirmed breast cancer diagnosis that underwent follow-ups at Seberang Jaya Hospital, Penang were recruited into this study. Clinical (age, ethnicity, stage, neo adjuvant therapy, family history of ovarian and breast cancers) and pathological (ER, PR, Her2 status, triple negativity, tumour grades and stages) characteristics of the breast cancers were obtained by retrospectively reviewing the medical records. Three mls of blood was taken from each subject and subjected to DNA extraction. These were then screened for germline mutations of *BRCA1* gene (exons 11, 13 and 16) for *BRCA2* gene (exons 10 and 11) using allele-specific PCR.

Results: The prevalence of *BRCA2*-only and combined *BRCA1* and *BRCA1* mutations were 28.6% (95% CI: 18%, 39.2%) and 71.4% (95% CI: 60.8%, 82.0 %), respectively. No wild-type *BRCA1* or *BRCA2 and BRCA1*-only mutations were observed in this study cohort. No significant associations were found between types of *BRCA* mutations (*BRCA2*-only mutations vs combined *BRCA1* and *BRCA2* mutations) and clinico-pathological characteristics of breast tumour. However, three *BRCA1* mutations (3232A>G (rs16941, exon 11), 3667A>G (rs16942, exon 11) and 4427T<C (rs1060915, exon 13) were significantly associated with a more advanced tumour size group (p values = 0.032, 0.049 and 0.043, respectively). Besides, 3232A>G (rs16941, exon 11) mutation was also significantly associated with higher risk of HER2-negative (OR 7.50 (95% CI: 1.439, 39.089), p value = 0.017) and triple negative breast carcinoma (OR 4.375 (95% CI: 1.193, 16.038), p value =0.042). No significant associations were found between *BRCA2* genotypes and clinico-pathological features of breast carcinoma.

Conclusion: Combined *BRCA1* and *BRCA2* mutations are the most prevalent types of *BRCA* mutations amongst females with early onset breast cancers, followed by *BRCA2*-only mutations. Three *BRCA1* germline mutations were found to be significantly predictive of a more advanced tumour size group whilst only one *BRCA1* mutation was significantly associated with HER2-negative and triple negative breast tumours. Nevertheless, further studies are warranted to address the unresolved issues encountered by this study.

CHAPTER ONE

INTRODUCTION

1.1 EPIDEMIOLOGY OF BREAST CANCER

Around the globe, cancer of the breast is the most frequently encountered cancer types in females. In the United States alone, it was estimated that 235,030 incident cases of malignant breast cancer, 62,570 carcinomas-in-situ and 40,430 deaths secondary to breast malignancy would occur in 2014 (Siegel *et al.* 2014). Breast cancer is implicated for the highest proportion of annual new cancer cases in female (29%) and second only to carcinoma of the lung and bronchus as the reason for cancer-specific mortality (15%) (Siegel *et al.* 2014). So far, the incidence of breast cancer has remained stable since 2005, after a sharp decline from 1999 to early 2005. This downward trend of breast cancer incidence may be attributable to the diminution of hormone replacement therapy (HRT) use among postmenopausal women (Ravdin *et al.* 2007). Nevertheless, the trend of breast cancer in young women is quite alarming. According to the analyses of Johnson, Chien & Bleyer (2013) which was based on Surveillance, Epidemiology and End Results (SEER) data obtained from 1976 to 2009, there is a statistically significant increase in the incidence of metastatic breast cancer among young women (defined as females in the age group of 25-39 years) in the United States (1.53 per 100,000 in 1976 to 2.53 per 100,000 in 2009, p value <0.001). Due to this alarming trend, it is critical to further the understanding and resolve the impending controversies with respect to the mechanistic and pathological basis of early-onset breast malignancy.

In Malaysia, the quality and contemporariness of breast cancer data provided by the National Cancer Registry (NCR) is still mediocre and of dismal standard. The only credible data on breast cancer incidence was provided by Dahlui, Ramli and Bulgiba (2011) who showed the declining trend of breast cancer incidence from 2003 to 2006 (46.2 per 100,000 vs 39.3 per 100,000). The only reported incidence of breast cancer in young females was by Pathy and colleagues (2011), who found a staggering 51 percent of females developed breast cancer at the age of 50 years or below. Nevertheless, such finding may be misleading since the analyses were based on the combined breast cancer cases in both Malaysia and Singapore and the different age limit used for defining early-onset breast cancer. The closest estimate, despite its outdated nature, is therefore the breast cancer statistics as reported by the NCR in 2007. According to NCR report, approximately 50% of breast cancer cases amongst women aged 50 or less and 16.8% of women under 40 years of age were affected by breast cancer (National Cancer Registry 2007). This estimate is thus not that far from what has been documented by Pathy *et al.* (2011). With regard to racial distribution of breast cancer cases, the Chinese have the highest age-standardized breast cancer incidence rate (59.7 per 100,000), followed by the Indians (55.8 per 100,000) and Malays (39.9 per 100, 000) (Yip, Mohd Taib, Mohamed 2006). Most of them were at the late stage of diagnosis (56.1% in stage III and IV at diagnosis) which was due to delayed diagnosis (Yip, Mohd Taib, and Mohamed, 2006). According to Bachok and associates (2011), use of alternative therapy, negative attitude towards treatment and false-negative diagnostic results are the associated factors for delayed breast cancer diagnosis

1.2 THE HISTOLOGICAL PERSPECTIVE OF BREAST CANCER

Breast cancer can be classified by several ways but the most common classification system identified two main types of invasive breast cancer; infiltrating ductal and infiltrating lobular carcinomas. Other tumour types such as medullary, tubular and mucinous carcinomas are considered less common and therefore will not be discussed in great details.

Invasive ductal carcinoma of no special type (NST) or not otherwise specified (NOS) is considered as the most common type of malignant breast pathology. It was estimated that this type of breast cancer was found in between 47% and 75% of all malignant breast cancer cases, depending upon the cohorts from whom data was obtained (Schnitt *et al*, 2003). The identification or confirmation of this tumour histology is usually made per exclusionem (diagnosis of exclusion). On gross appearance, invasive ductal carcinoma has a scirrhous or stellate shape with solid gray-white nodular mass that can either be well-demarcated or in poorly-circumscribed shape. On light microscopy, infiltrative mammary carcinoma of NST does not have particular features that may help pinpoint to its identification. The malignant epithelial cells appear to be organized in cords, tightly-cohesive nests, sheets and tubules that invade the fibrotic stromal part of the breast in a disorderly manner.

The second type of infiltrative breast cancer is the invasive lobular carcinoma. Classically, this tumour type is identified by its two most distinguishing characteristics; cytological features and invasive pattern. Invasive lobular carcinoma (ILC) has cytological characteristics that closely resemble lobular carcinoma-in situ: regular, flat-shaped nuclei with intracytoplasmic lacunae (Battifora, 1975). Besides,

its distinctive invasive pattern, targetoid growth pattern, makes it possible to be accurately and easily identified by histopathologist. There are also other variants of ILC such as solid, alveolar, mixed or pleomorphic types (Martinez & Azzopardi 1975). They are different to the classical ILC in terms of their nuclei shape and lack of targetoid growth features upon microscopic examination. This type of breast carcinoma has an intermediate prognosis (5-year survival of 70% to 80%), better than the prognostically-poor ductal carcinoma of no special type but much worse than the excellent prognosis of tubular carcinoma.

1.3 RISK FACTORS OF BREAST MALIGNANCY

Five major classes of risk factors have been identified as the determinants of breast cancer risk; types of dietary intake, exposure to ionizing radiation, use of exogenous hormone and reproductive factors, and genetic predisposition to breast cancer. Moderate-to-excessive alcohol intake is the most well-established factor for breast cancer (Chen *et al.* 2011). Other dietary factors for instance low fat and high fruit and vegetable intake have also been found to be generally protective against breast cancer (Linos & Willett 2007). Nevertheless, results from the multicentre Women's Healthy Eating and Living (WHEL) trial demonstrated no significant decrease of breast cancer recurrence in subjects who consumed low fat or high fruit and vegetable diet (Pierce *et al.* 2007). This observation is however at variance with the findings of the much larger Women's Intervention Nutrition Study (WINS trial) which showed a lowering of breast cancer recurrence risk among subjects randomized to low-fat dietary regime which may be attributed to moderate weight loss secondary to reduced calorie intake (Hoy *et al.* 2009). This discrepancy of findings, however, requires further investigations to solidly resolve the divergent conclusions made by the authors.

In regard to ionizing radiation, higher risk of breast malignancy is found in those exposed to either therapeutic (radiotherapy) or accidental exposure to radiation (e.g. Japanese survivors of atomic bomb, nuclear meltdown in Chernobyl). In the former, breast cancer risk has been found to be increased in recipients of extended field radiation therapy for Hodgkin's Lymphoma. The age at first radiotherapeutic exposure was found to be the principal factor in modulating breast cancer risk, with the highest and lowest risks were found among females who received mantle field radiotherapy at puberty and postmenopausal females, respectively (Swerdlow *et al* 2012, Ronckers, Erdmann & Land 2005).

The use of exogenous oestrogen in HRT has been singled out as one of the chief determinants of breast cancer. A combined analysis of data from the large multicentre Women's Health Initiative (WHI) study and other 51 additional studies revealed that the use of exogenous hormone was associated with a slight increase in risk of breast cancer development (Collaborative Group on Hormonal Factors in Breast Cancer 1997). Nonetheless, this small increase in breast cancer risk was counterbalanced by the decrease in colon and ovarian cancer risk of similar magnitude, resulting in no increase of overall breast cancer risk among HRT users (Colditz & Hankinson 2005). Other studies found the link between types and timing of HRT (oestrogen only vs combined, right after menopause vs delayed HRT institution) and breast cancer occurrence, with the highest risk found in those who were started on HRT soon after menopause occurs (Beral *et al*. 2011). All in all, with the observations made by Ravdin *et al.,* (2007), the use of HRT is associated with a slight but significant increase risk in breast malignancy especially in those who are above 50 years of age, used HRT right after menopause and users of oestrogen-only HRT.

Late age of menopause, early menarche, nulliparity and late age at first full-term birth (above 30 years of age) had been systematically found to be the major reproductive factors for breast cancer (Kelsey, Gammon & John 1993, McPherson, Steel & Dixon 2000). This finding is in coherence with the duration of lifetime exposure to oestrogen and the fact that breast cancer risk is higher in postmenopausal women who had oestradiol levels in the highest quartile (Farhat *et al.* 2011). Besides that, obese women are also at a greater risk of breast cancer due to higher circulating oestrogen and endogenous insulin levels than females with normal body mass index (BMI) (Hvidtfeldt, 2012). Therefore, this class of breast cancer aetiologies can be considered as the mechanistic "hinges" or the convergence point in the complex interplay of other risk factors within the web of causality for breast malignancy.

The roles of genetic predisposition in breast cancer development will be elucidated in the next chapter due to it being the main focus of this research initiative.

CHAPTER TWO

LITERATURE REVIEW

2.1 INHERITED BREAST CANCER SYNDROME

Inherited breast cancer syndrome has been defined as early-onset breast cancer (median age at diagnosis: 45 years, may occur as early as early 20s with an elevated risk for the whole lifetime of an individual), a nimiety of familial bilateral breast cancer cases, a larger occurrence of primary cancers in the hereditary breast and ovarian cancer (HBOC) spectrum and an autosomally-dominant feature of inheritance of cancer susceptibility genes (Lynch *et al.* 1994). This hereditary form of breast cancer differs from sporadic breast cancer in that the latter cases occur in females without positive family history of breast cancer affecting two consecutive generations (e.g parents, paternal and maternal aunts, uncles or grandparents, siblings) (Lynch *et al.* 2009). On the other hand, breast cancer with a positive family history of carcinoma of the breast involving at least one second or first-degree family members but lack of other features (eg variable age of onset) that may fit them in the inherited breast cancer syndrome category is classified as familial breast cancer (Lynch *et al.* 2009).

Due to the aggressive nature of hereditary breast carcinoma, genetic counselling and screening practices are compulsory for the management of this patient subgroup. So far, three different types of cancer susceptibility genes have been identified;

1) Highly-penetrant genes such as *Breast cancer susceptibility 1* (*BRCA1*), *Breast cancer susceptibility 2* (*BRCA2*), and *Tumour protein p53* (*TP53*)

2) Intermediately-penetrant genes, for instance *Checkpoint Kinase-2* (*CHEK2*) and *Ataxia Telangiectasia Mutated* (*ATM*)

3) Low penetrant genes (e.g *Fibroblast Growth Factor receptor-2* (*FGFR2*) and (*Thymocyte selection-associated high mobility group box*) *TOX*).

In the next section, *BRCA1* and *BRCA2* mutations will be discussed in greater details with exploration on the correlation between mutations in the respective genes with their associated clinicopathological characteristics.

2.2 *BRCA1* AND *BRCA2* GENES: THE CARETAKERS OF HUMAN GENOME

BRCA1, a tumor suppressor gene, is located in the long arm of chromosome 17 (17q21). It codes for a protein product that plays a critical role in repairing DNA double-strand breaks. It has 22 exons which encrypt for a protein of the size of 1863 amino acids. So far there were 1643 discrete mutations, polymorphisms and variants of this gene has been identified, of which 890 are discovered and reported in single individual only (ie. only one person was found to harbour each mutation, polymorphism or variant). *BRCA1* gene is one of the major causes for breast and ovarian cancer in females under 40, accounting for 5.3% and 5.7% of all breast and ovarian cancer cases in that subgroup, respectively (Ford & Easton, 1995). The main types of mutations found in *BRCA1* are frameshift, nonsense and splice mutations (Chen & Parmigiani, 2007). In a majority of cases, these mutations cause a non-functional *BRCA1* protein due to its premature shortening. For instance, a nonsense

mutation occurring in *BRCA1* results in one of the codons of *BRCA1* exons being replaced by a stop codon (Glu1541Stop, Gln1313Stop), leading to untimely truncation of the *BRCA1* protein (Futreal *et al.* 1994, Miki *et al.* 1994). This will in turn result in a dysfunctional or non-functional *BRCA1* protein that is unable to effectively repair double strand breaks in DNA (Miki *et al.* 1994).

Besides that, Easton and colleagues have discovered two distinct phenotypic variations of families affected by *BRCA1* germline mutation using linkage analyses: 1) families with high number of cases with ovarian carcinoma (84% of them have developed ovarian carcinoma by the age of 70 years), 2) those with relatively low number of ovarian carcinoma (ovarian carcinoma occurring in 32% of cases by 70 years of age). Furthermore, Gayther *et al*., (1995) also reported a statistically-significant association was found between the position of *BRCA1* mutation and breast to ovarian cancer ratio of incidence occurring in each of the family Mutations in the last third of the gene (at 3' position) are associated with a lower risk of ovarian cancer (Thompson *et al.* 2002, Gayther *et al.* 1995, Holt *et al* 1996).

The mechanism by which *BRCA1* protein assists in repairing double strand breaks in DNA is quite complex. BRCA1 primarily acts as the initial "sensor" of the existence of DNA double-strand break or gap which is then relayed to the other regulators of DNA-repair machinery. The end product of this DNA-repair signalling cascade is DNA repair by homologous recombination. A vivid explanation on the molecular structure of BRCA1 protein is therefore fundamental to understand its functional role as the custodian of genomic integrity in human (Venkitaraman 2014).

There are three main components of *BRCA1* proteins that may explain further its role in DNA double-strand break repair; 1) a Really Interesting New Gene

(RING) domain at N-terminus 2) *BRCA1* C-terminus (BRCT) domain at carboxyl (C) terminus, 3) a long central region between 170th and 1649th amino acid residues, flanked by the C and N termini (Venkitaraman, 2014). This long central region of *BRCA1* protein is hypothesized to be an intrinsically-disordered region, a view that has been supported by both in-silico and experimental models (Mark *et al.* 2005). The RING domain, which is further stabilized by its heterodimerization with *BRCA1*-associated ring domain-1 (*BARD1*), possesses E3 ubiquitin ligase activity (Roy, Chun & Powell 2012, Wu *et al.* 1996). The activation of *BRCA1-BARD1* ligase activity is usually induced by erroneous DNA replication or exposure to genotoxic stress (Greenberg *et al.* 2006). This *BRCA1-BARD1* ligase will then interact and polyubiquinate other proteins such as CtIP, histone H2A and H2AX (Thakar *et al.* 2010, Yu *et al.* 2006, Irminger-Finger & Jefford 2006). These proteins, especially CtIP, will then associate with *BARD1-BRCA1* complex via the C-terminus of *BRCA1* to form a *BRCA1*-CtIP complex (Wu *et al.* 2008). This *BRCA1*-CtIP complex will then initiate panoply of double strand break end resection to create overhung single-strand DNAs (ssDNA) that is vital for subsequent repair of double strand break by homologous recombination (Sartori *et al*. 2007, Limbo *et al.* 2007). Besides, the *BRCA1*-CtIP complex activity will also lead to cell cycle arrest at G2/M phase, during which the repairing of DNA double-strand break can be effectively implemented (Wu *et al.* 2007). These crucial roles of *BRCA1* RING domain in preventing tumourigenesis had been proven by Sankaran and co-workers (2006) who demonstrated missense mutations occurring inside *BRCA1* RING domain will lead to the abolishment of *BRCA1* ligase activity and thus culminating in overt tumour formation.

The other parts of *BRCA1* proteins also have great roles in initiating DNA double-strand break repair. The C-tandem repeat in BRCT domain mobilizes *BRCA1* protein to the damaged DNA sites (Venkitaraman, 2014). This initial recruitment is made feasible by the ability of C-terminus region of BRCT to bind to poly-ADP ribose (PAR) chains attached to the proteins at the DNA double strand break locations (Li *et al* 2013). This *BRCA1* recruitment to the damage site is subsequently augmented by the binding of *BRCA1* to the phosphopeptide motif of serine residue, which was generated by the activated DNA-damage-sensing tyrosine-kinase (Venkitaraman, 2014). Subsequently, using its BRCT domain, *BRCA1* protein engages and regulates various macromolecular complexes activity and assembly which function as DNA damage sensors and repair executors at DNA damage sites and stalled replication forks (Greenberg *et al*. 2006). Eventually, this *BRCA1*-associated macromolecular complex will displace 53BP1, a HR suppressant, from the DNA strand's broken end thus initiating the repair of DNA damage via homologous recombination during the G_2 phase of the cell cycle (Bouwman *et al* 2010, Bunting *et al.* 2010).

BRCA2 gene, on the other hand, is located on the long arm of chromosome 13 (13q12.3). It consists of 27 coding regions (exons) with a size of 84 kilobases (kb) (Ioan *et al*. 2009) So far, more than 450 new *BRCA2* mutations have been discovered which are disseminated over the whole span *BRCA2* gene (Ioan *et al*. 2009). Similar to *BRCA1*, founder mutations have also been characterized in several populations; for instance in Ashkenazi Jewish females, 185delAG and 6174delT are considered as among the first founder mutations and most prevalent *BRCA2* mutations ever described in such population (Struewing *et al.* 1995, Roa *et al.* 1996). Like *BRCA1, BRCA2* gene mutation is also associated with breast cancer at

early age and bilateral breast carcinomas. Interestingly, it is also associated with several types of cancer susceptibility syndrome such as the rare Fanconi anemia (FA) and Bloom syndrome (Patel *et al.* 1998). Its functional role as the caretaker of genomic integrity also lies in its ability to repair DNA double-strand breaks and thus preserving genomic fidelity during DNA replication (Ioan *et al.* 2009).

For it to function effectively, *BRCA2* proteins recruit RAD51, a DNA recombinase, as part of *BRCA2*-mediated DNA double-strand break repair machinery. *BRCA2* binds to the RAD51 protein and ships it to the DNA damage site at which it is subsequently released (Gudmundsdottir & Ashworth, 2006). Then, RAD51 protein initiates the formation of nucleoprotein filament on single-sranded DNA (ssDNA). This RAD51-ssDNA nucleoprotein complex then induces synapsis with the homologous sister chromatid which allows the exchange of DNA strand between the chromatids (Venkitaraman, 2014). This results in a successful DNA double strand break repair via the error-free homologous recombination (Saeki *et al.* 2006).

2.3 *BRCA1* AND *BRCA2* MUTATIONS: GLOBAL AND LOCAL PERSPECTIVE

The first population whose *BRCA1* and *BRCA2* mutation status was fully characterized is the Ashkenazi Jews. Struewing *et al.* (1995) discovered the high prevalence of 185delAG frameshift mutation in 0.9% (95% CI 0.4-1.8) Asheknazi Jewish subjects. A study by Roa and associates (1996) which utilized a large number of Ashkenazi Jewish individuals (n=3000) as the study subjects corroborated the findings Struewing *et al.* (1995). They found that the carrier frequency of *BRCA1* mutations to be at 1.09% which was further supported by the results obtained by

Offit *et al.*(1996) who demonstrated that 20% of early-onset breast cancer cases in Ashkenazi Jewish females was found to harbour 185delAG mutation. This confirms the founder effects of such mutation in Ashkenazi Jews.

In other populations, varying numbers of novel *BRCA1* mutations (with and without founder effects) were discovered. For instance, L63X and Q934X were found to be the most prevalent *BRCA1* mutations in Japanese population (Liede & Narod 2002). Based on haplotyping of *BRCA1*, both mutations were thought to be derived from the same ancestors, thus confirming them as founder mutations in Japanese population (Sekine *et al.* 2001). Among the Pakistanis, the highly-prevalent European *BRCA1* 4814del4 frameshift mutation has been found in 15% of individuals with breast carcinoma. Besides that, one *BRCA1* founder mutation (c.981_982delAT) had also been characterized in Southern Chinese breast cancer patients (Kwong *et al.* 2012) whilst 3478del5 and 5589del8 *BRCA1* mutations were found to exhibit founder effects for Northern Chinese breast cancer subjects (Karami & Mehdapour, 2013). For subjects of Scandinavian origins, 1675delA and 1135insC mutations were found to be prevalent and possess the founder effects in Norwegian and Danish populations (Thomassen *et al.* 2008). Therefore, the types of *BRCA1* mutation vary from one population to another, signifying profound ancestral effects in the evolution of *BRCA1* gene.

In our local setting, the prevalence of combined *BRCA1* and *BRCA2* mutations is 20% (Yip *et al.* 2009). For *BRCA1*, 14 sequence alterations had been documented and reported by Toh *et al.* (2008), of which 3 are novel findings. One deleterious germline *BRCA1* novel mutation (IVS3+2delT) was discovered by the researchers in one early-onset Malaysian Chinese breast cancer subject without obvious family history. One possible founder mutation (2846insA) has been

described by Lee *et al.* (2003) among Malay breast cancer patients residing in Singapore. A report by Thirthagiri *et al.* (2008) confirmed the heteregenous nature of *BRCA1* mutation; 14 novels *BRCA1* mutations were discovered with none of them were reported twice (ie. found in two unrelated individuals with early-onset breast cancer). Besides that, Lee and associates (2012) found similar frequencies of *TP53*, *BRCA1* and *BRCA2* mutations (5%, 6% and 11%, respectively). The authors suggested that the mutual coexistence of those three mutations, hence warranting *TP53* to be included the full genetic screening for breast cancer.

With respect to *BRCA2* mutations, as stated previously, 6174delT was the most common mutation found in Ashkenazi Jewish females with a prevalence rate of 1.5% (Oddoux *et al.* 1996). Therefore, both 185delAG and 6174delT can be considered as the most prevalent *BRCA1* and *BRCA2* mutations in Ashkenzi Jewish women. However, *BRCA2* mutation has generally a diminished penetrance than *BRCA1* counterpart (Chen and Parmigiani, 2007). Hence, the risk of breast early-onset breast carcinoma is lower in those who are positive for *BRCA2* mutations than subjects with *BRCA1* mutation (King *et al.* 2003).

In other populations, the frequency and types of *BRCA2* mutations differ remarkably. In an unselected series of breast carcinoma in Pakistani females living in Lahore and Karachi, Liede and associates (2002) found 3337C>T mutation as the commonest *BRCA2* mutation. This finding is corroborated by the subsequent discoveries by Farooq *et al.* (2011) who also found second new *BRCA2* mutation, 5057delTG. Both, however, are not founder mutations for this population. In the Far East, a founder *BRCA2* mutation, 5802delAATT, has been reported in Japanese population (Ikeda *et al*. 2001). Another founder *BRCA2* mutation, 7480C>T, was also discovered in Korean subjects by Seong and co-workers (2011). By far, Chinese

breast cancer subjects possess the highest number of founder mutations among far eastern population. Three founder mutations (3109C>T, 7436_7805del370 and 9097_9098insA) have been reported by Kwong and colleagues (2012) in Southern Chinese population. This signifies that Far Eastern population has a higher probability of inheriting the defective copies of *BRCA2* mutation which arise from a single ancestor. This is hardly surprising due to genetic inbreeding secondary to a higher prevalence of intra-racial marriage (or consanguineous marriage) practised by Far Eastern population than in other populations. Besides, similar pattern can also be observed in other populations that also practise inbreeding due to isolated geographical region and cultural similarities. For instance, nearly all known *BRCA2* mutation in Scandinavian populations are founder mutations (Jara *et al.* 2006, Soegaard *et al.* 2008) As a result, the aberrant *BRCA2* mutation is preserved to the extreme which result in a higher number of founder mutations in Far Eastern females with breast carcinoma than their South Asian (Pakistani, Indian and Bangladeshi) counterparts.

In Malaysia, four *BRCA2* mutations have been documented so far; 4859delA, 4265delCT, 1342C.A, 490 delCT (Toh *et al.* 2008). The first two *BRCA2* mutations are also the most common *BRCA2* mutations among Filipinos breast cancer females (De Leon 2002). This finding is indeed very fascinating since such shared *BRCA2* mutations between two closely-related populations but living in two distinct geographical locations have been reported in other studies as well. For instance, the founder *BRCA2* mutation, 999del5, has been reported in three ancestrally-related populations from three different countries (Iceland, Denmark and Finland) (Gunnarson *et al.* 2008, Hartikainen *et al.* 2007, Soegaard *et al.* 2008). Hence, these observations merit further research to ascertain whether these two *BRCA2* mutations

are the founder mutations for both Malays and Filipinos individuals. As a corollary, it may also provide the concrete evidence that both Malays and Filipino breast cancer sufferers come from the same ancestral lineage. This may result in the construction of phylogenetic tree for *BRCA2* mutation which will enable the current cancer researchers to design a highly-specific anti-cancer pharmacotherapy which will be effective in at least improving the survival breast cancer subjects in these two populations. Nevertheless, such endeavour has yet to be taken place and further works are required before such aspiration can be fully realized. As a summary, Table 2.1 demonstrates the current "state-of-affairs" with regard to *BRCA1* and *BRCA2* mutations worldwide.

Table 2.1: Types of *BRCA1* and *BRCA2* mutations in selected populations of different ancestries (adapted from Karami &Mehdipour, 2013)

Countries	***BRCA1* mutations**	***BRCA2* mutations**	**Founder mutations**
Scandinavian / Viking			
Finland	c.5095C>T 4216-2ntA–>G 5370C>T	4088insA, c.68-80insT c.793+34T>G 999del5 6503delTT	4216-2ntA>G 5370C>T 999del5 6503delTT
Sweden	3172ins5 2594delC 1806C>T 1201del11	4486delG	3172ins5 2594delC 1806C>T, 1201del11 4486delG
Denmark	3172ins5 1201del11 1675delA 1135insC	2594delC, 5382insC 3829delT Q563X 3438G>T, 1675delA, 999del5	**Iceland/Denmark:** (999del5) **Swedish/Danish founder:** (3172ins5,1201del11,) **Danish specific:** 2594delC,5382insC, 3829delT, and Q563X
Iceland	G5193A Exon 13 & 22 del 2804delAA	999del5	999del5, G5193A
Malay Archipelago			
Malaysia	c.2845insA, 4427T>C, 2846insA, 2201C>T 4956A>G, 3668A>G, 2731C>T, 3232A.G, 3667A.G, exon 3 dup	4859delA, 4265delCT, 1342C>A 490 delCT	**Malaysian/Singaporean founder:** c.2845insA (Malay) **Malaysian/Filipino founder:** 4859delA?
Philippine	5454delC	4265delCT 4859delA	5454delC, 4265delCT, 4859delA
Singapore	c.2845insA, Exon 13 dup	Exons 4–11 dup	c.2845insA

Table 2.1 continued...

Countries	*BRCA1* mutations	*BRCA2* mutations	Founder mutations
Indonesia	-	6775G>T, p.Glu2183X, c.2699_2704delTAATG	c.2699_2704delTAAATG
Eastern Orientals			
China	3478del5, 5589del8, 1100delAT, 2778G>A, 3552C>T, exon 10 dup, 5,273G>A c.470 471delCT,	7883delTTAA c.2808 2811delACAA, c.3109C>T, c.7436 7805del370, c.9097 9098insA	**Hong Kong:** 5589del8, 1100delAT **Southern China:** c.3109C>T, c.3109C>T, c.7436 7805del370, c.981 982delAT,
Japan	c.307T>A	5802delAATT, 8732C>A, c.2835C>A	c.188T>A, c.2800C>T c.2835C>A, c.307T>A, 5802delAATT
Korea	509C>A, c.2333delC, c.4065 4068delTCAA 3746 3747insA 5199G>T	c.7480C>T, 1627A.T 3972delTGAG, 7708C.T	c.7480C>T
Indian ancestry			
India	185delAG 2983C>A, 3450delCAAG c.3548A>G, c.-26G>A, c.317-54C>G	—	185delAG
Pakistan	4627C>A (22%), 4184del4(15%), 185delAG, 2080insAIVS14−1G>A (11%), 2041insA 4284delAG(8%), 3889delAG 2388delG (7%)	3337C>T (50%), 5057delTG (50%)	4627C>A, 185delAG, 185insA
Sri Lanka	c.3086delT, c.5404delG	—	—

2.4 ASSOCIATION BETWEEN *BRCA1/BRCA2* MUTATIONS AND CLINICOPATHOLOGICAL CORRELATES

2.4.1 General overview

It has been vastly documented that the *BRCA1* mutation is associated with early-onset breast cancer, higher tumour grades and negative for hormonal receptors (i.e no oestrogen, progesterone and HER2/neu (ERBB2) receptors (Karp *et al.* 1997, Atchley *et al.* 2008). Despite all the above features are associated with dismal prognosis, the results from various studies are discrepant with several of them reporting grimmer prognosis (Brekelmans *et al.* 2006) whilst others documenting similar prognostic outlook (Rennert *et al.* 2007).

Distinct clinicopathological differences have been documented between *BRCA1* and *BRCA2*-associated breast cancer. Kwong *et al.* (2009) showed the presence of *BRCA1* mutations was significantly associated with bigger size of tumours (higher proportions of stage T2 and T3 tumours) and negative estrogen receptor (ER) status. No significant differences were found in terms of proportion of triple negative breast tumours between *BRCA1* and *BRCA2* mutation groups. These results are, however, at variance with the ones reported by Atchley *et al.*, (2008) who reported a significantly higher proportion of triple negative breast cancer in those harbouring *BRCA1* mutation. Apart from that, they also reported higher nuclear grades, as assessed by Black's nuclear grading system, in those with *BRCA1* mutation when compared to *BRCA2*-associated cancer or non-carriers of both mutations (85.4% vs 56.5% vs 38.4%, p value <0.001). Interestingly, Atchley and associates (2008) also reported the use of hormonal replacement therapy is associated with higher number of *BRCA2*-associated tumour (*BRCA1* vs *BRCA2*

mutations: 30.0% vs 9.3%, p value = 0.04). These findings are consistent with Turchetti *et al.* (2000) who established significant association between the presence of *BRCA1* mutations and higher proliferative index (*BRCA*+ vs *BRCA*-: 100% vs 46%, p value = 0.017), greater proportion of poorly differentiated tumour (*BRCA*+ vs *BRCA*-: 100% vs 44%, p value = 0.044) and greater percentage of negative estrogen receptor status (*BRCA*+ vs *BRCA*-: 100% vs 52%, p value = 0.020). This insuperable evidence provides the undisputable basis that the *BRCA1* mutation is an adverse prognostic factor in breast cancer patients.

With respect to the incidence of primary ductal carcinoma in situ (DCIS), lesser proportion of DCIS was found among *BRCA1* positive cases. A research endeavour led by Hamilton and associates (2004) at the Nottingham Breast Institute found none (0%) of the carriers of *BRCA1* mutations were of DCIS histology whilst, on the other hand, 36% of those harbouring *BRCA2* mutation were of positive for DCIS (p value = 0.010). This finding is corroborated by those of Marcus *et al.* (1996) who demonstrated *BRCA1* mutation was significantly associated with a lower proportion of lobular carcinoma in situ (LCIS) and atypical lobular hyperplasia (ALH) when comparison was made with *BRCA2* mutation (2.7% vs 23.5% , p value = 0.010). The observations made by the Europen Breast Cancer Linkage Consortium further cemented the fact that DCIS and LCIS are rare entities in *BRCA1* positive subjects (Breast Cancer Linkage Consortium 1997). This evidence, hence, confirms the scarcity of LCIS and DCIS in *BRCA1* mutation carriers..

There are also striking dissimilarities between *BRCA1* and *BRCA2* carriers with respect to the gene expression profiles of tumour tissues. Based upon microarray data of breast cancer tissues, mammary carcinomas can be further subdivided into 4 distinct gene expression characteristics; basal-like, luminal-like

(both luminal A and luminal B), HER2/neu overexpressing and those resembling normal breast tissues (Hedenfalk *et al.* 2001, Sorlie *et al.* 2003). The majority of *BRCA1*-associated breast cancers have basal-like features which are distinguishable by its high expression of cytokeratins 5, 6, 14 and 17, P-cadherin, EGFR, osteonectin, caveolin 1 and others (Honrado *et al.* 2006). This is in tandem with the high incidence of medullary and infiltrating carcinoma with medullary characteristic in *BRCA1* carriers, both exhibit basal-like characteristics and triple negativity for ER, PR and HER2 status (Jacquemier *et al.* 2005, Rodriguez-Pinilla *et al.* 2007, Honrado *et al.* 2006). On the other hand, most *BRCA2* mutation carriers are of luminal-like group (Larsen *et al.* 2013, Bane *et al.* 2008).

2.4.2 Survival status in *BRCA1* and *BRCA2* mutation carriers

With respect to overall survival status and other survival parameters (eg metastasis-free survival, progression-free survival etc), the evidence, as described briefly in the previous section, is conflicting. Despite possessing poorer prognostic elements (aneuploidy, high rate of cell division, poorer degree of differention), no significant difference was shown between Ashkenazi Jewish females who carried *BRCA1* mutation and those who were non-mutation carriers in terms of hazard of death (Hazard ratio (HR) 0.76 (95% CI 0.45, 1.30), p value = 0.31) (Rennert *et al.* 2007). Besides, they also showed no statistically-significant higher hazard of death among the carriers of *BRCA2* mutation when compared to the non-carriers (HR 1.31 (95% CI 0.80, 2.15) , p value = 0.28). These results are in contradiction to those of Kurian *et al.* (2010) who showed lower survival probability of reaching 70 years of age in *BRCA1* mutation carriers than *BRCA2* counterparts (53% and 71%, respectively). Nevertheless, the findings of Goodwin and associates (2012) negated what had been published by Kurian *et al.* (2010). They reported no significant differences were

noted between *BRCA1* mutation carriers and sporadic breast cancer cases with respect to hazard of death or distant recurrences of breast tumours (HR_{death}: 0.99 (95% CI 0.62, 1.59), $HR_{recurrences}$: 0.83 (95% CI 0.51,1.83)). Apart from that, upon analysis by multiple cox proportional hazard regression model, no significant impact of *BRCA2* mutation was found on overall survival and distant breast cancer recurrences. Therefore, further studies are necessary to provide answers to this unsolved enigma.

2.4.3 Mechanistic relationship between *BRCA1* mutations and hormonal receptor status

There are a number of mechanisms through which *BRCA1*-related tumours cause marked deficiencies in the expression of estrogen and progesterone receptors. Firstly, the genetic alteration of *BRCA1* may promote underexpression of estrogen receptors. This is evident from the observations of Hosey *et al.,* (2007) who demonstrated a 5.4-fold (95% CI 2.6-fold, 40.1-fold, p value = 0.002) lowering of mean estrogen receptor 1 (*ER1*) gene expression in *BRCA1*-related breast cancer when compared to sporadic breast cancer. Besides that, another mechanism explaining the relationship between *BRCA1* mutations with negative estrogen receptor status has been proposed by Fan and colleagues (1999). They theorized that *BRCA1* mutation may perturb the intracellular signalling mediated by ligand-activated estrogen receptor alpha (*ER*-α). This in turn will lead to the blocking of activation of AF2 portion of *ER*-α which may cause the repression of subsequent *ER*-α expression. These findings had been corroborated by Ma *et al.* (2006) who showed that *BRCA1* causes the downregulation of *p300* expression, a known transcriptional co-activator of *ER*-α gene.

Besides that, another interesting hypothesis has been put forward by a Liu *et al.,* (1996) who demonstrated *BRCA1* mutation is associated with disturbed normal differentiation of mammary tissues which may alter the phenotypes of breast cancer cells and increase the number of cells at risk for tumourigenesis. This supports the notion that *BRCA1*-associated breast cancer cells may raise from distinct progenitor cells other than the ones which are *BRCA1*-negative, resulting in ER positivity in the former and ER negativity in the latter. This is thus compatible with the previous findings that the presence of *BRCA1* mutation is associated with basal-like carcinoma, a subtype of breast cancer that lacks estrogen and progesterone receptors. Besides that, King *et al.* (2004) also established *BRCA1* haploinsufficiency is associated with marginally-insignificant reduced mean progesterone receptor expression when compared to *BRCA2* cases (26.1 vs 9.6, p value = 0.06). Nevertheless, no experimental findings have so far conclusively elucidated how interaction between types of *BRCA1* mutations and other crucial genetic mutations may give raise to ER and PR-negative breast tumours. More molecular works need to be done to supply these mechanistic lacunae of breast cancer pathogenesis with the necessary and verifiable experimental results.

2.4.4 *BRCA1* / *BRCA2* mutation and clinicopathological correlates: Local perspectives

For our local setting, it was Yip *et al.* (2009) who first successfully described the link between *BRCA1 / BRCA2* mutation status and triple negative breast cancer. They demonstrated significantly higher proportions of ER and PR-negative breast cancer in *BRCA1*-associated breast cancer than *BRCA2* ones in Malaysian setting (ER 93.3% vs 33.3%, p value =0.002; PR 100% vs 50%, p value =0.007). No significant difference was found with respect to HER2 negativity between *BRCA1*

and *BRCA2*-positive breast cancer (84.6% vs 80.0%, p value =0.774). Nevertheless, the results on HER2 receptor might be flawed since all indeterminate results (Immunohistochemistry grade 2) were discarded from analyses and Fluorescence In Situ Hybridization (FISH) was not employed to determine the indeterminate HER2 results. Besides, only a small number of subjects possessing either *BRCA1* or *BRCA2* mutations were included in the analyses (total frequencies of *BRCA1* and *BRCA2* mutations positive are 16 and 15, respectively). Apart from that, the prevalences of genotypes for each newly-found *BRCA1*/BRCA2 mutations and the measures of associations (e.g such as odds ratio and 95% confidence intervals for odds ratio) between types of *BRCA1* and *BRCA2* mutatons with clinicopathological correlates have not been reported by Yip *et al.* (2009). Besides that, the effects of other well - known determinants of hormone receptor status and histological types of breast tumours such as use of hormone replacement therapy, nulliparity and increasing age of first childbirth (Yang *et al.* 2011) were also not statistically controlled for using multivariable regression. Consequently, additional studies are needed to clearly characterize the relationship between clinicopathological features of breast tumours and types of *BRCA1* and *BRCA2* mutations in Malaysian setting.

2.5 PROPHYLACTIC INTERVENTIONS FOR *BRCA1* AND *BRCA2* MUTATIONS CARRIERS

There are several prophylactic treatments that have been advocated for those who are positive for *BRCA1* or *BRCA2* mutations. The strategies for breast cancer prophylaxis can be divided into two; medical and surgical approaches. With regard to the former, Narod and associates (2000) have found that the use of tamoxifen confers protection against contralateral mammary carcinoma in those harbouring *BRCA1* mutations (OR 0.38, 95% CI 0.19, 0.74) and *BRCA2* mutations (OR 0.63,